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The natural product peiminine represses colorectal carcinoma tumor growth by inducing autophagic cell death

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ABSTRACT

Autophagy is evolutionarily conservative in eukaryotic cells that engulf cellular long-lived proteins and organelles, and it degrades the contents through fusion with lysosomes, via which the cell acquires recycled building blocks for the synthesis of new molecules. In this study, we revealed that peiminine induces cell death and enhances autophagic flux in colorectal carcinoma HCT-116 cells. We determined that peiminine enhances the autophagic flux by repressing the phosphorylation of mTOR through inhibiting upstream signals. Knocking down ATG5 greatly reduced the peiminine-induced cell death in wild-type HCT-116 cells, while treating Bax/Bak-deficient cells with peiminine resulted in significant cell death. In summary, our discoveries demonstrated that peiminine represses colorectal carcinoma cell proliferation and cell growth by inducing autophagic cell death.

1. Introduction

Autophagy is an evolutionarily conserved pathway that delivers cellular contents to lysosomes for degradation [1]. It is an important regulatory process in eukaryotic cells that plays a critical function in the maintenance of cellular homeostasis and antagonization of metabolic stress [2,3]. Double-membrane vesicles, autophagosomes, are formed during autophagy, and these engulf cytoplasm and organelles. This is followed by fusion with lysosomes, and then the proteins and organelles are finally degraded and the obtained products are recycled as new building blocks for biomolecule synthesis [4]. Autophagy is extensively involved in numerous physiological and pathological processes, including adipogenesis, starvation and tumorigenesis, etc. [5–9]. Being a "double-edged sword", autophagy provides "recycled" energy for rapid cell growth under biological stress, on one side [10]. While on the other, the uncontrolled degradation of cytoplasmic contents by excessive autophagy is likely to be lethal for cells [11]. For instance, activation of autophagy contributes to the tolerance of nutrient deprivation in colorectal carcinoma [12]. However, dimethyl cardamonin (DMC)-induced autophagy was demonstrated to significantly suppress cell proliferation through a G2/M phase cell-cycle delay and it causes cell death in colorectal carcinoma cell lines [13].

Autophagic cell death, also called type II cell death, is an alternative programmed cell death (PCD) pathway other than apoptosis (type I cell death), which has also been validated in colorectal carcinoma [13–16]. Autophagy exists commonly during cell death under stress, but not all cell death where autophagy is present can be referred to as autophagic cell death, as cell death in many circumstances is accompanied by protective autophagy. Cells deficient in Bax and Bak, proapoptotic members of the Bcl-2 family that are essential for releasing apoptosis proteins, do not undergo apoptosis when exposed to apoptotic stimuli [17–19]. When alternative pathways were activated, these cells experienced cell death. Studies revealed that numerous double-membrane vesicles were formed in Bax/Bak-deficient cells that undergo cell death [20]. Moreover, the distribution of GFP-LC3B puncta was observed, which confirmed the formation of autophagosomes that implicated autophagic cell death [20].

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Peiminine is a natural compound extracted from *Fritillaria thunbergii*, which is widely used in Traditional Chinese Medicine for the treatment of various diseases, including cancer [21]. In this study, we showed that peiminine induced phenomenal autophagy activation and significant tumor growth repression in colorectal carcinoma cells. Our data revealed that a peiminine treatment significantly induces autophagy by activating the autophagy flux through the AMPK and PI3K/Akt pathways. By determining the cell viability level in ATG5 knock-down cells and Bax/Bak-deficient HCT-116 cells, we demonstrated that peiminine induced cell death was independent of apoptosis, at least partially. Together, our result revealed the autophagy-inducing capability of peiminine and indicated that the tumor repressive effect was mediated by autophagic cell death.

2. Materials and methods

2.1. Cell culture and transfection

The colorectal carcinoma cell line HCT-116 was cultured with Dulbecco's modified Eagle's medium supplied with 10% fetal bovine serum in a 5% CO_2 humidity incubator at 37 °C. Cells were subcultured when they reached 90% confluence. A chemically synthesized siRNA duplex was transfected into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

2.2. RNA isolation, reverse transcription and quantitative real-time PCR

Post transfection (24 h) cells were collected and after a wash in 1 × PBS they were supplied with RNAiso (Takara). The total RNA was then isolated and quantified following the manufacturer's protocol. First strand cDNA was reverse transcripted using a random primer by M-MLV (Takara). Quantitative real-time PCR was performed using SYBR Master Mix (TOYOBO) with specific primers on an AB7300 real-time PCR system (Applied Biosystems). All expressions were normalized by GAPDH.

2.3. Immunoblot assay

Total cell lysate was collected and electrophoresed on a 4–12% bis-acrylamide gel at 100 V for two hours. The proteins were transfer to a nitrocellulose membrane with a 300 mA current for 90 min. The primary antibodies used for Western blot were LC3B (Sigma, L7543), mTOR (CST, #4517), p-mTOR (CST, #5536), p-ULK1 (S555) (CST, #5869), pan-Akt (CST, #4685), p-Akt (S473) (CST, #4060), PTEN (CST, #9188), p-PTEN (CST, #9549), p-AMPK (CST, #4188) and GAPDH (CST, #5174).

2.4. Cell proliferation assay

HCT-116 cells were seeded in 96-well plates with 2000 cells per well and treated with a series of peiminine (Chinese National Institute for Food and Drug Control, NIFDC) solutions with final concentrations of 0, 100, 200 or 400 μM. A CCK-8 cell proliferation assay was performed according to the manufacturer's protocol (Dojindo).

2.5. Confocal microscopy

GFP-LC3B stable expression HeLa cells were seeded and cultured in a flask overnight. A gradient concentration of peiminine was added to the cells with final concentrations of 0, 50, 100 and 200 μM. Cells were observed with an Olympus FV1000 (Olympus) confocal microscope 24 h after treatment.

2.6. Flow cytometry

Cells were treated with serial concentrations of peiminine and DMSO solvent, as the negative control, and then cultured in DMEM supplied with 10% FBS for 24 h. The cells were then collected and stained with propidium iodide and Annexin V according to the manufacturer's guidelines (BD Biosciences) and analyzed using a BD Influx™ (BD Biosciences) flow cytometer.

2.7. Immunohistochemistry

Tumors were isolated from xenograft mice after euthanasia and fixed in 5% paraformaldehyde at room temperature for 48 h. Selected samples were embedded in paraffin and sectioned; they were then stained with hematoxylin and eosin; and exposed to cleaved caspase 3 (Biosynthesis Biotech, bs-0087R) and LC3B (Sigma, L7543). The primary antibody LC3B and cleaved caspase 3 were used at the ratio of 1:100. The sections were then mounted for histological analysis.

2.8. Animal experiments

Four-week old female BALB/c nude mice were purchased from Guangdong Experimental Animal Center (Guangzhou, China) and kept in a pathogen-free environment. Approximately 1×10^6 HCT-116 cells were underarm injected into each mouse. One week post tumor implantation, all mice were randomly divided into two groups ($n = 5$): the vehicle (control) group received intraperitoneal (i.p.) injections of saline every two days while the peiminine group received i.p. injections of 3 mg/kg peiminine every two days. At the end of the experiment (two weeks after tumor implantation), all mice were euthanized, tumors were isolated and the weight of each animal was recorded.

2.9. Statistical analysis

All results were expressed as means ± SD from at least three independent experiments. A Student two-tailed *t* test was applied for statistical analysis.

3. Results

3.1. Peiminine induces autophagy in HCT-116 cells

To investigate the autophagy inducing ability of natural compounds derived from Traditional Chinese Medicine, several natural products, including polydatin, peiminine, forsythin and macranthoidin B, were screened in a previous study by assessing the autophagy activation potential in GFP-LC3 stable expression HeLa cells. By comparing the GFP puncta number in drug administration cells and the control, we discovered peiminine was the best autophagy activator among these candidates (Figure S1). Therefore, we applied a gradient dosage of peiminine (50, 100 and 200 μM) in HeLa-GFP-LC3 cells with a control solvent and observed an increase in the GFP puncta in a dose-dependent manner (Fig. 1A). We quantified the GFP-LC3B positive cells according to puncta number per cell, and categorized the cells into three classes (cells with less than 10 puncta per cell, 10–30 puncta per cell and more than 30 puncta per cell). The results showed that under the 50 μM peiminine treatment, the proportion of cells that had 10 or more puncta per cell was significantly elevated (Fig. 1C). To investigate the autophagy inducing capability of peiminine in colorectal

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